IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	§	
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PECKER et al	§	
	§	
Serial No.: 09/776,874	§	
	§	
Filed: February 6, 2001	§	Group Art Unit: 1652
	§	
For: Polynucleotide Encoding A Polypeptide Having Heparanase Activity and Expression of Same in Genetically Modified Cells	§	
	§	
	§	Attorney Docket: 01/21603
	§	
Examiner: R. G. Hutson	§	

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313

DECLARATION UNDER 37 U.S.C. SECTION 1.131

Sir:

I, Iris Pecker, declare as follows:

1) I am the Iris Pecker who is an inventor named in the above-identified subject invention.

2) I understand that claims 97 to 99 are rejected as allegedly not enabled by the specification. While the current Office Action alleges that the specification does not enable a heparanase protein that includes a polypeptide that is at least 70% homologous to SEQ ID NO:10, by rejecting claims 98 and 99, the Office Action is also alleging that the specification does not enable a heparanase protein that includes a polypeptide that is at least that is at least 80% or even 90% homologous to SEQ ID NO:10.

3) It is the intent of this declaration to provide three objective scientific reasons why the skilled artisan could have identified working embodiments that fall within the scope of claims 97, 98 and 99 with routine experimentation. First, the genetic drift or variation within the naturally occurring species of heparanase proteins, as shown in Figure 17 of the subject application and as described further below, provides a roadmap for variations including point mutations. Further variation can be gleaned from the secondary structure of the protein, as shown in Figure 19, which can provide for example the location of the proton donor and likely nucleophiles. Second, the skilled artisan's knowledge of protein chemistry allows prediction of preserved biological properties so long as amino acid substitutions are conservative in their nature. Finally, the secondary structure of heparanase, as shown in Figure 19, would allow the skilled artisan to predict areas of non-criticality where even non-conservative substitutions may be introduced.

3) As stated in my declaration submitted previously in the prosecution of this case, I have analyzed the alignment data shown in Figure 17 of the subject application. In my opinion, it provides ample guidance to the skilled artisan on how to make active heparanase variants that include polypeptides at least 70% homologous with SEQ ID NO:10, and certainly variants that are at least 80% or 90% homologous with SEQ ID NO:10.

4) For example, residues 85 to 106 of human heparananse (SEQ ID NO:10) are identical to the corresponding residues of the variant mouse and rat heparanases shown in Figure 17. It is important to note that these variants share less than 70% homology. By contrast, residues 23 to 36 have 11 residue differences. Similarly, comparing rat and human heparanase, residues 129 to 138, for example, have 8 differences among the 10 residues, with 9 of 10 differences among mouse and human at this region. With such guidance, the skilled artisan would know to not vary residues 85 to 106 and to vary one or more residues among residues 23 to 36 and/or 129 to 138, especially with a similar amino acid residue substitution (e.g., hydrophilic). The skilled artisan could even further use the guidance of the subject

specification to replace one or more amino acid residues in SEQ ID NO:10, especially in these highly variable regions, with those corresponding residues found in mouse or rat heparanase.

5) Looking at heparanase protein more broadly, residues 49 to 109 make up 61 residues. Comparing mouse and human region at this region, there are only 10 of 61 changes. Comparing rat and human at this region, there are also only 10 of 61 changes. This is therefore a very conserved region, one that the skilled artisan would likely not vary, at least as a starting point, in trying to obtain additional heparanase homologs.

6) The predictions discussed above have become true. For example, the conserved region of residues 49 to 109 was confirmed to be the 8 kDa unit of active heparanase. By contrast, variable regions 23 to 36 and 129 to 138, discussed above, are not part of either the small or large units of mature heparanase.

7) Moreover, I point out for the first time in this case that Figure 19 of the subject application provides even further guidance. Figure 19 shows the secondary structure prediction for heparanase using computer assistance. The portions depicted as "H" are helical, and the portions depicted as "E" are extended beta strand structures.

8) Still further, the glutamic acid residue of heparanase, predicted as the proton donor, is marked with an asterisk in Figure 19. Given the relative location of the proton donor and the predicted secondary structure of the protein, the glutamic acid residue that functions as the nucleophile is most likely at position 343 or 396 (see underlined residues in Figure 19, and page 105, lines 20-22 of the subject specification).

9) In summary, given the wealth of information in the subject specification, particularly from figures 17 and 19, and the scientific technology available for exploiting this information, at the time of the subject invention, the skilled artisan could have made heparanase variants that included a polypeptide at least 70% homologous with SEQ ID NO:10, and certainly at least 80% or 90% homologous with SEQ ID NO:10, without undue experimentation.

10) As also outlined in my earlier declaration, I have also reviewed an analysis of the heparanase preparation taught by Fuks et al. (U.S. Pat. No. 5,362,641; hereinafter "Fuks"). This analysis shows that the heparanase of Fuks was inextricably mixed with a significant amount of at least six other proteins: PAI-1, Nexin-I, Vimentin, Grp94/endoplasmin, FLT receptor and Tryptase.

11) Indeed, the amount of these non-heparanase proteins present was so significant, that antibodies to one of these proteins (PAI-1) were elicited, while antibodies to heparanase could not be.

12) There are two reasons why Fuks failed to achieve sufficient purity of their preparation so as to be able to elicit anti-heparanase antibodies. First, heparanase is "sticky." Second, the amount of heparanase found in cells is extremely low. For these two reasons, it would not have been obvious to purify the preparation of Fuks according to the methods of Fuks sufficiently so as to have a preparation that is pure enough to elicit anti-heparanase antibodies.

13) One reason why it is so difficult and unobvious to purify the heparanase preparation of Fuks to the point where anti-heparanase antibodies can be elicited is because heparanase protein is very sticky to sugars. Consequently, heparanase is sticky to separation columns. This is known as non-specific affinity.

14) Proteins can only be separated via one factor at a time, such as size, pH, etc. The stickier a protein is to a column, the less it will be able to be separated because the stickiness factor will always interfere with the primary separation factor that one is using. The result is a broader band in the separation column, which means the protein is still meshed with other proteins.

15) Indeed, Fuks subjected their preparation to a further purification cycle according to the methods they taught. The results of their analysis of this preparation were provided to us and showed that this preparation contained many proteins, as much as nine of them in amounts sufficient for amino acid micro sequencing; in other words very high amounts.

16) The stickiness of heparanase is exacerbated by the fact that it is difficult and unobvious to acquire a sufficient amount of this protein. Indeed, the level of heparanase in most cell lines, including SKH is low. For example, heparanase is not detected in an extract of 10^6 SKH cells by anti-heparanase antibodies which can detect quantity in the nanogram range. Heparanase activity could not be detected in SKH cells using a DMB assay, which can also detect quantity even in the nanogram range. Furthermore, in the very sensitive ECM assay, which can detect a protein in the picogram range, more than 10^6 cells are required to detect heparanase activity.

17) In fact, Fuks needed 20 liters of culture to start with to arrive at their preparation and still failed to achieve sufficient purity to elicit anti-heparanase antibodies. This is because the fraction of heparanase in the total cell extract is extremely small, as explained above, and the purification procedure to eliminate all other cellular proteins must include several chromatographic steps. When purifying a protein, which comprises a small fraction of the protein mixture, a significant loss of the protein of interest is expected in each chromatographic step. This is specifically true for heparanase, which is a non-covalently bound dimer that is easily disintegrated. The ECM assay, used to follow the enzyme during the purification procedure is very sensitive and allows the detection of even picograms of protein. Following such a multi-step procedure one expects to yield an extremely small amount of protein, which would be too small even for evaluation of purity and obviously not sufficient to elicit anti-heparanase antibodies.

18) Using Fuks' procedures, nine contaminants could be identified. Although one could continue and employ the additional column suggested by Fuks, which would decrease the amount of contaminants, it would also decrease the amount of heparanase to the point where it could not elicit anti-heparanase antibodies. Moreover, the additional column step would still not separate all proteins which were co-eluted up to this point.

19) In stark contrast to the method taught by Fuks, the methods of the subject invention were able to avoid all of the difficulties discussed above and therefore result is a preparation that is pure enough to elicit anti-heparanase antibodies.

20) Specifically, using the heparanase gene, heparanase can be over-expressed. This yields an extract which is a-priori enriched with heparanase. The loss of heparanase during chromatography is therefore insignificant. Moreover, the availability of the sequence allows prediction of the protein pI, and other physicochemical characteristics that enable the design of a rational and efficient purification procedure. Using such an expression system as the source of protein, due to the high expected yield of heparanase, contaminants that may be co-eluted with heparanase would comprise a negligible proportion of the purified protein.

I declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willfully false statements are punishable by fine or imprisonment under 18 U.S.C. Section 1001 and that any such statement may jeopardize the validity of the subject application or any patent issued thereon.

Is Pecker

Dr. Iris Pecker

28/1/09

Date

DECLARATION OF ISRAEL VLODAVSKY

I am presently employed as researcher in the Hadassah-Hebrew University Hospital (Department of Oncology, Jeruasalem 91120, Israel). I chair the Tumor Biology Research Unit of the Sharett Oncology Institute of the Hadassah Hospital. I am a full professor in the Faculty of Medicine of the Hebrew University School of Medicine. I received my Ph.D degree from the Weizmann Institute of Science (Rehovot, Israel) in 1975, worked as a post-doctoral fellow in UCLA and UCSF and was a visiting Professor at Harvard Medical School (Children's Hospital, Boston). In 1980 I established a Tumor Biology Research Laboratory at the Hadassah-University Hospital. My research focuses on basic and clinical aspects of tumor metastasis and angiogenesis, with emphasis on cell interaction with the extracellular matrix, heparin-binding growth factors and heparin/heparan sulfate-degrading enzymes. Since the beginning of my carrier, I have published 247 scientific articles in highly regarded journals and books, and have presented my achievements at more than 90 international scientific conferences. I am a member of several international scientific societies and important local committees, and was awarded the 1997 prize for a distinguished Israeli scientist in medicine. For the last 20 years I have been engaged in the research of heparanase and heparan sulfate and have published over 70 papers in the field (see enclosed curriculum vitae).

I hereby declare the following:

Heparanase has a specific, well characterized and unique catalytic activity known for over 20 years. Over the years, heparanase was partially purified from a variety of mammalian sources. Heparanase is defined as a GAG hydrolase which cleaves heparin and heparan sulfate (both are sulfated) at the b1,4 linkage between glucuronic acid and glucosamin. Heparanase is an endolytic enzyme and the average

product length it generates is 8-12 saccharides. The other known heparin/heparan sulfate degrading enzymes are b-glucuronidase, a-L iduronidase and a-N acetylglucosaminidase. These three enzymes are exolytic enzymes, each of which cleaves a specific linkage within the polysaccharide chain and generates disaccharides. These issues are further addressed below.

There are three sources of reports regarding false heparanase antibodies as follows:

An anti PAI-1 antibody, which is described in U.S. Pat. No. 5,362,641 (D3), was produced in an attempt to elicit anti-heparanase antibodies. This antibody was elicited by a PAI-1 contamination in a purified sample of heparanase, as was observed by peptide analysis.

Identification of this antibody as an anti PAI-1 antibody is discussed in U.S. Patent No. 5,968,822. Page 11, line 18 to page 12, line 2, recite in this respect that:

> Several years ago we prepared rabbit polyclonal antibodies directed against our partially purified preparation of human placenta heparanase. These antibodies, referred to in U.S. Pat. No. 5,362,641, were later found to be directed against plasminogen activator inhibitor type I (PAI-1) that was co-purified with the placental heparanase. These findings led to a modification of the original purification protocol to remove the PAI-1 contaminant.

Another false anti-heparanase antibody was generated against the chemokine CTAP III, a protein that was reported to possess heparanase-like activity. These antibodies were generated by the group of Prof. Ledbetter as described in Hoogewerf et al. J Biol Chem 17;270(7):3268-77, 1995) and were donated to other researchers as reported by Kosir et al. (J Surg Res. 67(1):98-105, 1997, page 99, materials and methods, right column, western blot).

CTAP III is a low molecular weight chemokine, which has no homology to heparanase from human placenta, SK-hepatoma, platelets (Hullet et. al. Nat. Med

5(7): 803-809, 1999) and SV40 transformed fibroblasts (Toyoshima and Nakajima J. Biol. Chem. 274(34):24153-24160, 1999) which were all purified and cloned recently and correspond to the amino acid sequence set forth in SEQ ID NO. 2 of the present application.

Because CTAPIII and heparanase, as defined by SEQ ID NO:2 of the present application, share no sequence homology, these antibodies are irrelevant. In addition, it was declared and it is now accepted by the scientific community, as is recited below, that CTAPIII was erroneously thought to be heparanase.

The third false anti-heparanase antibody was generated in the laboratory of Prof. Nicolson and was first reported by Jin et al. (Int J Cancer. 45(6):1088-95, 1990). This group isolated a 96 kDa mouse protein and used a peptide derived from the N-terminus of the partially purified protein to generate polyclonal as well as monoclonal antibodies. These antibodies detect a 96 kDa protein, which is obviously different from the placental heparanase referred to in the instant application and which was later isolated from other tissues as currently reported by several other groups. These antibodies were used by several research groups in collaboration with either one of the authors of the original paper (Marchetti et al. Cancer Res. 56(12):2856-63, 1996, Marchetti and Nicolson, Adv Enzyme Regul. 37:111-34, 1997, Mollinedo et al. Biochem J. 327(3):917-23, 1997).

In 1994, Vouge et al. (Int. J. Cancer 56:286-294, 1994) pointed out the fact that the antibodies claimed to detect heparanase actually detect GR94/endoplasmin, a previously cloned and characterized murine heat shock protein. The sequence and the molecular weight were in perfect agreement with those reported for the 96 kDa murine heparanase isolated by Nicolson's group. Later on, the mis-identification of the heparanase enzyme and consequently the antibodies generated against it was

admitted and accepted by the scientific community. The late papers (1996, 1997) still referring to these antibodies, as heparanase specific, are obscure. There is no doubt, however, that those antibodies do not recognize heparanase.

Kosir et al. provided yet another source of confusion in the field of heparanase, which only now has been resolved. Kosir et al. disclose anti-CTAP III antibodies. CTAP III is a platelet derives chemokine to which heparanase activity was erroneously attributed in the past. No sequence homology is observed between CTAP III and heparanases derived from, for example, placenta and from hepatoma cells (SEQ ID NO:2 of the present application, Vlodavsky et al. Nat. Med. 5(7):793-802, 1999, Kosir et al. Biochem Biophys Res Commun. 261(1):183-7, 1999) and as was later reported, from platelets (Hullet et al. Nature Medicine 5(7):803-809, 1999) and from SV40 transformed fibroblasts (Toyoshima and Nakajima J. Biol. Chem. 274(34):24153-24160, 1999).

The antibody used by Kosir at al. (J Surg Res. 67(1):98-105, 1997) was donated thereto by Dr. Ledbetter. According to lines 15-16 of the "Western blot" section, production of these antibodies was described in Hoogewerf et al. (J. Biol. Chem. 270(7): 3268-77, 1995). In this paper Hoogwerf et al., a research group from Upjohn Company, Kalamazoo, Michigan, describe the identification of CXC chemokines (the CTAPIII family) as heparan sulfate degrading enzymes. The antibodies described in the paper were raised against CTAPIII, which shares no sequence homology with the 50 kDa heparanase. Moreover, in a recent paper the same group from Pharmacia and Upjohn, Inc. retracted from their earlier statement regarding the heparanase activity of CTAPIII (Fairbank et al. J Biol Chem 274(42): 29587-29590, 1999) page 29590, right column, last paragraph of the discussion. They state that:

> Finally, an earlier report from this laboratory suggested that heparanase was a post-translationally modified form of a CXC chemokine, namely CTAPIII (7). We have not been able to confirm this observation, nor have others who have purified and characterized human heparanase

In this paragraph they refer to their previous paper, Hoogewerf et al. (J Biol Chem 1995 Feb 17;270(7):3268-77).

It is accepted today by the scientific community that CTAPIII is not a heparanase or a precursor thereof.

I turn now to a detailed discussion of the references cited by the Examiner in the recent Official action and which are said to teach anti-heparanase antibodies.

As is evident from the background section of the instant application, the response filed herewith (see in particular the concluding remarks, and the arguments) and this declaration, the need for anti-heparanase antibodies is well recognized for many years and many unsuccessful attempts were made to obtain such antibodies. D3 clearly recognizes a particular need for anti-heparanase antibodies, however, recognizing a need does not qualify as anticipation. The need for anti-heparanase antibodies is indeed recognized by the art. However, this need was not fulfilled, nor does D3 fulfill this need.

Thus, D3 fails to teach anti-heparanase antibodies, and certainly does not teach or suggest the many important antibody assays described in the present application.

Marchetti et al. briefly mention the use of antibody developed against heparanase. No description of an antibody source, preparation or characteristics is provided. No data is shown regarding such an antibody. The authors refer to a manuscript in preparation. I failed to find any later publications which provide this data. In a similar paper published by Marchetti and Nicolson (Adv Enzyme Regul.

37:111-34, 1997) the same "results" are briefly described with the remark "data not shown" (see, page 127, the paragraph just before the discussion). In a more recent paper which discusses heparanase regulation in human melanoma and where Marchetti is the last author, the results are based solely on activity measurements (Walch et al. Int. J. Cancer 82:112-120, 1999). Mollinedo et al. (Biochem J. 327(3):917-23, 1997) report localization of heparanase using the monoclonal antibody and refer on page 918 (materials and methods, antibodies) to Marchetti at al. (Cancer Res. 56:2856-2863, 1996). Mollinedo et al. show immunoblots where the heparanase antibody detects a 96 kDa protein (page 920, Figure 2).

It is my knowledge that this antibody was generated in the laboratory of Prof. Nicolson and was first reported by Jin et al. (Int J Cancer. 45(6):1088-95, 1990). This group isolated a 96 kDa mouse protein and used a peptide derived from the N-terminus of the partially purified protein to generate polyclonal as well as monoclonal antibodies. These antibodies detect a 96 kDa protein, which is obviously different from placental heparanase and which was later isolated from other tissues as currently reported by several groups. These antibodies were used by several research groups in collaboration with either one of the authors of the original paper (Marchetti et al. Cancer Res. 56(12):2856-63, 1996, Marchetti and Nicolson, Adv Enzyme Regul. 37:111-34, 1997, Mollinedo et al. Biochem J. 327(3):917-23, 1997). In 1994, Vouge et al. (Int. J. Cancer 56:286-294, 1994) pointed out the fact that the antibodies claimed to detect heparanase actually detect GR94/endoplasmin, a previously cloned and characterized murine heat shock protein. The sequence and the molecular weight were in perfect agreement with those reported for the 96 kDa murine heparanase isolated by Nicolson's group. Later on, the mis-identification of the heparanase

enzyme and consequently the antibodies generated against it was admitted and accepted by the scientific community.

Interestingly, Prof. Nicolson has abandoned heparanase research and does not take part in the major progress achieved during the recent years. Dr. Nakajima is a researcher at Novartis, a company that published recently the cloning of heparanase, with Nakajima as a last author (Toyoshima and Nakajima, J. Biol. Chem. 274(34):24153-24160, 1999). The published sequence is identical to SEQ ID NO:2 listed in the present application and the molecular weight of the purified protein is of 50 kDa.

Thus, clearly these allegedly "anti-heparanase" antibodies were not specific for heparanase, unlike the antibodies of the present invention.

Thus, it is clear that the antibodies according to the present invention are novel and are also inventive over the background art, none of which taught or suggested a truly specific molecular probe against heparanase.

I hereby certify that the above facts and statements are true and complete, to the best of my knowledge.

Israel Vlodavsky

Prof. Israel Vlodavsky

Date: June 12, 2003